

utilized to generate humanized antibodies in which the complementarity determining regions (CDR's) from a donor mouse or rat immunoglobulin are grafted onto human framework regions (for example, EPO Publication No. 0239400, incorporated herein by reference). The process is called "humanization", or "CDR-grafting". The original concept of humanization describes the direct grafting of CDR's onto human frameworks, reducing the non-human sequences to less than 5%, and thereby the HAMA and HACA responses. However, direct replacement of framework sequences without further modifications can result in the loss of affinity for the antigen, sometimes as much as 10-fold or more (Jones et al., *Nature*, 321:522-525, 1986; Verhoyen et al., *Science*, 239:1534-1536, 1988). To maintain the affinity of the CDR-grafted or humanized antibody, substitutions of a human framework amino acid of the acceptor immunoglobulin with the corresponding amino acid from a donor immunoglobulin at selected positions will be required. The positions where the substitution takes place are determined by a set of published criteria (US 5,85,089; US 5,693,762; US 5,693,761; incorporated herein by reference). However, the presence of murine amino acids within stretches of human framework sequences can be immunogenic in the generation of new T- and B-cell epitopes. Moreover, the identification of the proper framework amino acids to be replaced can sometimes be difficult, further reducing the chances of success in humanization without significant impacts on the specificity and affinity of the humanized antibody.

New and improved means for producing re-engineered immunoglobulin with reduced or eliminated immunogenicity while maintaining the specificity and affinity of the parent antibody are therefore needed. Preferably, the re-engineered immunoglobulin should contain no FR amino acid substitutions from the parent antibody, which can be a likely source of immunogenic epitopes for T- or B-cells. However, the approach also offers flexibility in the sequence design where few murine residues or a stretch of murine sequences can be included in the final design, with the ultimate goal of reducing immunogenicity while maintaining specificity and affinity of the resultant

SUMMARY OF THE INVENTION

The present invention relates to novel methods for re-engineering immunoglobulin chains having generally one or more complementarity
5 determining regions (CDR's) from a donor immunoglobulin and portions of framework sequences from one or more human, or primate immunoglobulins. The preferred methods comprise first dividing the framework sequences from immunoglobulins of all species into compartmentalized subregions of FR1, FR2, FR3, and FR4, according to the Kabat Database (Kabat et al. Sequences
10 of proteins of immunological interest. Maryland: US Department of Health and Human Services, NIH, 1991), and comparing the individual FR's, instead of the whole framework, in the variable region amino acid sequence subregions of the parent immunoglobulin to corresponding sequences in a collection of human, or primate immunoglobulin chains, and selecting the
15 appropriate human or primate FR's with the highest degree of homology to replace the original FR's of the parent immunoglobulin (framework- or FR-patching). The human FR's can be selected from more than one human or primate immunoglobulin sequences. A collection of human or primate immunoglobulin sequences can be obtained from different databases (for
20 example, Kabat database, National Biomedical Research Foundation Protein Identification Resource, Brookhaven Protein Data Bank, internet, etc.). The individual FR sequences selected from human or primate immunoglobulins will typically have more than 60% homology to the corresponding parent FR sequences. Although high overall homology will be an important criteria for
25 selecting the FR's for patching, lesser homology FR's will be used if the homology of sequences directly flanking the CDR's or at loop positions where contact(s) with the antigen binding site is (are) determined experimentally or predicted via computer modeling. The parent immunoglobulin whose FR's are to be patched may be either a heavy chain or light chain. A patched light
30 and heavy chain can be used to form a complete FR-patched immunoglobulin or antibody, having two light/heavy chain pairs, with or without partial or full-length human constant regions.

The individual FR's chosen for patching a parent immunoglobulin chain (applies to both heavy and light chains) should:

- 5 (1) preferably have amino acid sequences immediately adjacent to the CDR's identical to that of the parent immunoglobulin chain;
- (2) have amino acid sequences immediately adjacent to the CDR's conservatively similar in structure to, if not completely identical to, that of the parent immunoglobulin chain;
- 10 (3) preferably have identical amino acid at corresponding FR position of the parent immunoglobulin predicted to be within about 3 Å of the CDR's (or the effective antigen-binding site) in a three-dimensional immunoglobulin model and capable of interacting with the antigen or with the CDR's of the parent or FR-patched immunoglobulin;
- 15 (4) have amino acid conservatively similar in structure to amino acid at corresponding FR position of the parent immunoglobulin predicted to be within about 3 Å of the CDR's (or the effective antigen-binding site) in a three-dimensional immunoglobulin model and capable of interacting with the antigen or with the CDR's of the parent or FR-patched immunoglobulin.
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Each of the heavy and light chains of the FR-patched immunoglobulin will typically comprise FR's sourced from one or more human or primate immunoglobulins according to any one or all of the selection criteria. The FR-patched heavy and light chains of the present invention, when combined into an intact antibody, antibody fragment, or antibody-based derivatives (for example single-chain antibody, diabodies, etc.), will be substantially non-immunogenic in humans and retain substantially the same affinity and properties (for example internalization upon binding) as the parent immunoglobulin to the antigen. These affinity levels should vary within the range of 4-fold, and preferably within about 2 fold of the parent immunoglobulin's original affinity to the antigen.

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Similar principles apply to re-engineer, or patch, parent immunoglobulins of one species with the FR's from a different species. People skilled in the art of protein and/or molecular engineering will be able to adopt the design and principle of the present invention to produce FR-patched immunoglobulins, or derivatives thereof. Once designed, there exist a variety of techniques in constructing the FR-patched immunoglobulin sequence, for example, by site-directed mutagenesis, and gene-synthesis. The assembled FR-patched sequences will be subcloned into expression vectors containing the appropriate immunoglobulin constant heavy and light chains for transfection in producer cell lines. Different cell systems can be used for the production of the FR-patched immunoglobulins, including bacterial, yeast, insect, and mammalian cells.

Alternatively, the immunoglobulins can be produced in the milks of transgenic or transomatic animals, or as stored proteins in transgenic plants. The present invention offers an improved and novel methods, that are relatively easy (no need to identify important FR amino acid interacting with the CDR's) and highly flexible (freedom to match, and change if necessary, individual FR's) in generating immunoglobulins with reduced or eliminated immunogenicities without sacrificing binding affinity and the likelihood of introducing new T- and B-cell epitopes resulting from the introduction of parent immunoglobulin's framework amino acids into the human FR's. The FR-patched antibodies will be suitable for human use in treating a variety of disease, either used singly or repeatedly, at low (less than 10 mg/m²) or high (more than 100 mg/m²) doses, in naked forms or as fusion or chemical conjugates, used alone, or in conjunction with other immunoglobulins or treatment modalities.

DETAILED DESCRIPTION OF THE FIGURES

Fig. 1A and Fig. 1B. Amino acid sequences (single-letter code) of the heavy chain (VH)(A) and light chain (VL)(B) variable regions of the murine anti-CD22 antibody, RFB4. CDR's are boxed.

Fig. 2A and Fig. 2B. Comparison of the compartmentalized framework sequences (FR's) of the heavy chain (A) and light chain (B) variable regions of RFB4, with the different human FR's of the highest homology. The FR1, FR2, FR3, and FR4 are indicated. The CDR's are boxed. The bracketed italic next on the left of the FR sequence indicates the source of the human FR. Amino acids in the human FR's that are different from that of the corresponding murine FR's are in bold.

Fig. 3A and Fig. 3B. The final designed sequences (single-letter code) of the heavy chain (A) and light chain (B) variable regions of the FR-patched antibody, hpRFB4. CDR's are boxed. Amino acids in the human FR's that are different from that of the original murine FR's are in bold.

Fig. 4. SDS-PAGE analysis of purified cRFB4 and hpRFB4 under both reducing and non-reducing conditions.

Fig. 5. Flow cytometry analyses on the binding specificity and affinity of cRFB4 and hpRFB4 on Raji cells. An irrelevant antibody was used as a control.

Fig. 6. Competition binding assay comparing the binding affinity between cRFB4 and hpRFB4.

Fig. 7A and Fig. 7B. Amino acid sequences (single-letter code) of the heavy chain (A) and light chain (B) variable regions of the murine anti-CD20 antibody, 1F5. CDR's are boxed.

Fig. 8A and Fig. 8B. Comparison of the compartmentalized framework sequences (FR's) of the heavy chain (A) and light chain (B) variable regions of 1F5 with the different human FR's of the highest homology. The FR1, FR2, FR3, and FR4 are indicated. The CDR's are boxed. The bracketed *italic* next on the left of the FR sequence indicates the source of the human FR. Amino acids in the human FR's that are different from that of the corresponding murine FR's are in bold.

Fig. 9A and Fig. 9B. The final designed sequences (single-letter code) of the heavy chain (A) and light chain (B) variable regions of the FR-patched antibody, hp1F5. CDR's are boxed. Amino acids in the human FR's that are different from that of the original murine FR's are in bold. Murine FR's not replaced by human sequences are underlined.

Fig. 10. Amino acid sequence of an alternative design of FR-patched variable regions for 1F5 (Alternative Design). CDR's are boxed. Human framework amino acids that differ from that of the corresponding murine frameworks are in bold.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention aims to establish novel approaches in the design of immunoglobulin with high degree of homology to human or primate sequences through a process named "framework (FR) patching". The FR-patched immunoglobulin (patched immunoglobulin thereafter) will have substantially reduced, or eliminated immunogenicity when used in human, and carry most or all of the characteristics of a human immunoglobulin such as the ability to target specific antigens, and effector functions (for example, 10 complement fixation, ADCC, etc.), while maintaining the specificity and affinity of the parent immunoglobulin against a specific antigen. The patched immunoglobulin will comprise a heavy and light chain, of which, the respective variable region will contain sequences representing FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4, according to Kabat's classification (Kabat et al., op. cit.). At least one of the four FR's of each parent immunoglobulin chain containing one or more complementary determining regions (CDR's) will be replaced, or "patched" with, a corresponding human or primate FR. When two or more FR's of the parent immunoglobulin chain are to be replaced, they can be patched with corresponding FR's either from the same 20 human or primate immunoglobulin, or from different human or primate immunoglobulin within the same subgroup or in different subgroups, or from a combination of human and primate immunoglobulins. The patched immunoglobulins will be expressed in appropriate host system for large-scale production at typical pharmaceutical margins, and used in humans at appropriate formats or combinations to treat or detect a wide range of human 25 diseases.

To ensure a better understanding of the present invention, several definitions are set forth. As used herein, an "immunoglobulin" refers to a protein 30 consisting of one or more polypeptides substantially encoded by immunoglobulin genes. A typical immunoglobulin protein contains two heavy chains paired with two light chains. A full-length immunoglobulin heavy chain is about 50 kD in size (approximately 446 amino acids in length), and is encoded by a heavy chain variable region gene (about 116 amino acids) and a

constant region gene. There are different constant region genes encoding heavy chain constant region of different isotypes such as alpha, gamma (IgG1, IgG2, IgG3, IgG4), delta, epsilon, and mu sequences. A full-length immunoglobulin light chain is about 25 Kd in size (approximately 214 amino acids in length), and is encoded by a light chain variable region gene (about 110 amino acids) and a kappa or lambda constant region gene. Naturally occurring immunoglobulin is known as antibody, usually in the form of a tetramer consisting of two identical pairs of immunoglobulin chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the effector functions typical of an antibody.

Immunoglobulin may be in different forms, either naturally occurring, chemically modified, or genetically-engineered, such as Fv (Huston et al., Proc. Natl. Acad. Sci. USA. 85:5879-5833; Bird et al., Science 242:423-426, 1988), diabodies, mini-antibodies, Fab, Fab', F(ab')₂, bifunctional hybrid antibodies (Lanzavecchia et al., Eur. J. Immunol. 17:105, 1987) (See, generally, Hood et al., "Immunology", Benjamin, NY, 2nd ed. 1984; Hunkapiller and Hood, Nature 323:15-16, 1986).

The variable region of both the heavy and light chain is divided into segments comprising four framework sub-regions (FR1, FR2, FR3, and FR4), interrupted by three stretches of hypervariable sequences, or the complementary determining regions (CDR's), as defined in Kabat's database (Kabat et al., op. cit.), with the CDR1 positioned between FR1 and FR2, CDR2 between FR2 and FR3, and CDR3 between FR3 and FR4. Without specifying the particular sub-regions as FR1, FR2, FR3 or FR4, a framework region, as referred by others, represents the combined FR's within the variable region of a single, naturally occurring immunoglobulin chain. As used herein, a FR represents one of the four sub-regions, and FR's represents two or more of the four sub-regions constituting a framework region. The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody is the combined

amino acid residues may have to be incorporated in the framework region of the acceptor immunoglobulin. There is a set of criteria for selecting a limited number of amino acids within the acceptor immunoglobulin for conversion into donor sequences, as published in a series of publications (US 5,85,089; 5 US 5,693,762; US 5,693,761; incorporated herein by reference). The humanized immunoglobulins may or may not contain constant regions. A humanized heavy chain immunoglobulin is a humanized immunoglobulin comprising a corresponding human heavy chain constant region, and a humanized light chain immunoglobulin is a humanized immunoglobulin 10 comprising a corresponding human light chain constant region. A humanized antibody is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin.

A successful humanized antibody will have to have the following characteristics:

- 15 (1) significantly reduced, and preferably eliminated, immunogenicity resulting from the humanized sequences, allowing multiple injection of the humanized antibody for human uses;
- 20 (2) minimally perturbed immunoreactivity including specificity and affinity (within 3-fold) against the original antigen;
- 25 (3) capable of inducing human effector functions such as complement fixation, complement-mediated cytotoxicity, antibody-dependent cell cytotoxicity, etc.

Direct grafting of donor CDR's onto human acceptor framework without further sequence modifications, will likely result in substantial loss of antigen 30 affinity. Although the introduction of selected donor amino acids to acceptor framework regions will somehow rectify the problem, and most of the time, improve affinity, however, the approach is tedious, requiring sometimes the assistance of computer modeling in identifying the appropriate framework amino acid to mutate, and lack flexibility in the choice of acceptor human 35 frameworks in an all-or-none mode. Most importantly, it is likely to introduce

potential new immunogenic epitopes by retaining parent "donor" residues in the human "acceptor" framework.

5 The present invention addresses these problems and creates a novel approach with increased flexibility and simplicity in generating a FR-patched antibody that is not immunogenic or is low in immunogenicity, yet having retained most or all of the original affinity against a specific antigen, as in the parent antibody. Since most of the immune responses against a chimeric or humanized immunoglobulin will be directed against epitopes in the variable
10 regions, without intending to be bound by theory, the principle by which the invention comes about will be illustrated by, but not limited to, using the variable region as the example.

15 There exist at least two kinds of epitopes contributing to the immunogenicity against a protein. The so-called "T cell epitopes" are short peptide sequences released during the degradation of proteins within cells and subsequently presented by molecules of the major histocompatibility complexes (MHC) in order to trigger the activation of T cells. For peptides presented by MHC class II, such activation of T cells can then give rise to an antibody response by
20 direct stimulation of B cells to produce such antibodies. A detailed analysis of the structure of a humanized variable region reveals the unavoidable existence of stretches of potentially immunogenic CDR's. These CDR's physically and functionally compartmentalize the rest of the framework sequences into four sub-regions, namely, the FR1, FR2, FR3, and FR4 (Kabat et al., op. cit.).
25 Since T cell epitopes are linear continuous short peptides, the presence or absence of such epitopes in each FR compartments should have no correlation to each other, whether the different FR's are derived from the same or different frameworks. The introduction of donor framework residues to the acceptor framework region using the humanization approach of Queen et al.
30 (US 5,85,089; US 5,693,762; US 5,693,761; incorporated herein by reference) will have the possibility of generating new, immunogenic T cell epitopes, resulting in the elicitation of immune responses against the humanized antibody, particularly antibody responses against the idiotypic region formed by the donor CDR loops. It is uncommon to have between 3 to 7 donor amino

acids incorporated into each humanized immunoglobulin chain, greatly increasing the chances of emergency for new T cell epitopes.

5 Similarly, these donor-derived residues embedded within the human framework can form new immunogenic B-cell epitopes recognizable by antibodies. While it is well-established that re-introduction of donor residues to the acceptor framework is important in maintaining the original antigen affinity of the humanized immunoglobulin, ideally, it would be preferable if humanization can be accomplished by direct grafting of donor CDR's onto
10 acceptor framework without additional modification and loss of affinity.

The present invention provides a new approach in reducing or eliminating the immunogenicity of immunoglobulins whose affinity against the specific antigen is maintained within three fold of its original level. The approach is
15 flexible, versatile, simple, and does not usually require sophisticated computer modeling analysis (although it does not preclude its being used). It deals with the problem of reciprocal relation between reducing immunogenicity and maintaining affinity in humanizing an antibody with the previous and available methodologies. Using an immunoglobulin variable region as
20 example, a set of criteria and principles will be followed in FR-patching the sequence. The criteria may be used singly, or when necessary in combination, to achieve reduced or eliminated immunogenicity, and the desired affinity or other characteristics.

25 In humanizing an immunoglobulin variable region by FR-patching, the parent immunoglobulin amino acid sequences are compartmentalized into FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4 according to the classification of Kabat et al. (opt. cit.). Each of the compartmentalized FR's will be dealt with separately and used to align with the corresponding FR segments found in all
30 databases, available either in the public domain, commercial entities, or private possession (for example the Kabat database, opt.cit.; the National Biomedical Research Foundation Protein Identification Resource). An immunoglobulin can be patched with FR's from more than one immunoglobulin sources. Preferably, human FR segments with the highest

- degree of homology (>60%) to the corresponding parent FR's will be used. However, amino acids in the FR's adjacent to one or more of the 3 CDR's in the primary sequence of the immunoglobulin chain may interact directly with the antigen (Amit et al., Science, 233:747-753, 1986, which is incorporated
- 5 herein by reference) and selecting these amino acids identical to the human FR's with lesser homology will be used according to the criteria set forth below.
- A human FR1 will be used when it has the highest homology to the parent
- 10 FR1, preferably 100%, at three or more amino acids immediately adjacent to CDR1.
- A human FR2 will be used when it has the highest homology to the parent FR2, preferably 100%, at three or more amino acids at both ends immediately
- 15 adjacent to the flanking CDR1 and CDR2.
- A human FR3 will be used when it has the highest homology to the parent FR3, preferably 100%, at three or more amino acids at both ends immediately adjacent to the flanking CDR2 and CDR3.
- 20 A human FR4 will be used when it has the highest homology to the parent FR4, preferably 100%, at three or more amino acids immediately adjacent to CDR3.
- 25 In case human FR's with 100% homology at three or more amino acids adjacent to the CDRs cannot be identified, FR's with the closest homology at these positions containing conservatively similar amino acids, such as, gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr; will be selected.
- 30 Preferably, human FR's whose amino acids at positions known to be close to, or have interactions with the CDR's/antigen binding site (Chothia and Lesk, J. Mol. Biol. 196:901, 1987; Chothia et al., Nature 342:877, 1989, and Tramontano et al., J. Mol. Biol. 215:175, 1990, all of which are incorporated

herein by reference), either based on computer modeling (see Levy et al.,
Biochemistry 28:7168-7175, 1989; Brucoleri et al., Nature 335:564-568,
1988; Chothia et al., Science 233:755-758, 1986, all of which are incorporated
herein by reference), crystal structure, published information, or prior
5 experience, which are identical, or conservatively similar to that of the parent
FR's will be selected.

FR-patching does not preclude the introduction of parent amino acids at
corresponding positions of a patched FR where necessary, or the inclusion of
10 FR's in the immunoglobulin from different species such as different primates,
murine, etc, when available databases fail to produce a satisfactory FR that
meet the above criteria. The primary goal is to produce antibodies with
reduced, preferably, eliminated immunogenicity without substantial loss of
affinity. The approach increases the chances of success in this regard, with
15 significant improvements over other methods in terms of flexibility,
simplicity, and ease of operation.

FR-patched antibodies carrying human constant sequences will be able to
induce human immune effector functions such as complement-mediated
20 cytotoxicity (CM) or anti-body-dependent cellular cytotoxicity (ADCC), upon
binding to the target antigens. Moreover, when injected in human for
therapeutic or diagnostic purposes, antibodies patched with human FR's are
expected to be non-immunogenic, i.e., will not elicit antibody responses
against the injected protein, allowing for multiple injections into human
25 patients if necessary for achieving maximum clinical benefits. Non-human
antibodies have been reported to have significantly shorter circulation half-
lives than that of human antibodies (Shaw et al., J Immunol. 138:4534-4538,
1987). The patched antibodies, carrying mostly human sequences, will
presumably have an extended half-life reminiscent to naturally occurring
30 human antibodies.

In the construction of a FR-patched immunoglobulin, sequence design for the
variable regions of the immunoglobulin will be done using the criteria and
principles illustrated above. The designed FR-patched variable region

sequence will be assembled using a variety of standard recombinant techniques well known to those skilled in the art. Preferably, the designed sequence, usually of a size of about 350 base pairs, will be gene-synthesized (Leung et al., *Molecular Immunol.* 32:1413-1427, 1995; Daugherty et al., Nucl. Acid Res. 19:2471-2476; DeMartino et al., *Antibody Immunoconj. Radiopharmaceut.* 4:829, 1991; Jones et al., *op. cit.*, all of which are incorporated herein by reference), or the individual FR's can be introduced to replace the parent FR's by methods of site- or oligonucleotide-directed mutagenesis (Gillman and Smith, *Gene* 8:81-97, 1979; and Roberts et al., *Nature* 328:731-734; both of which are incorporated herein by reference).

The DNA segment encoding the FR-patched immunoglobulin will be joined to DNA sequences encoding the human heavy and light chain regions in DNA expression vectors suitable for bacterial propagation and expression in different host cells. There are a variety of DNA vectors suitable for expression in a variety of host cell systems. Appropriate DNA vectors can be chosen for the expression of the FR-patched immunoglobulins. Typically, a suitable expression control DNA sequence is linked operably to DNA segments encoding the immunoglobulin chains. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. The sequence encoding the FR-patched heavy and light immunoglobulin chains can be incorporated into one single DNA expression vector, or into separate heavy and light chain expression vectors. In the latter case, host cells will have to be simultaneously incorporated with both vectors in order to produce a FR-patched antibody with the properly paired heavy and light chain polypeptides. In general, a leader sequence allowing the transportation of the immunoglobulin polypeptide into the Golgi apparatus for later secretion is included at the N-terminal end of each immunoglobulin chain for expression in eukaryotic host cells. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the humanized light chains, heavy chains, light/heavy chain dimers or intact antibodies,

binding fragments, single chain antibody (sFv), diabodies, or derivatives thereof, or other immunoglobulin forms may follow (see Beychok, Cells of Immunoglobulin Synthesis, Academic Press, NY, 1979, which is incorporated herein by reference).

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It is a well-known fact that there are different human constant regions for the heavy and light chains. A particular isotype will have specific effector characteristics that can be chosen for use for different purposes. Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat op. cit. and WP87/02671). The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to the predetermined antigens, such as CD22 and CD20, for example, and produced by well known methods in any convenient mammalian source including, mice, rat, rabbits, or other vertebrate, capable of producing antibodies. Suitable source cells for the constant region and framework DNA and secretion, can be obtained from a number of sources such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," sixth edition, 1988, Rockville, MD, USA, which is incorporated herein by reference).

DNA expression vectors containing the coding sequences for the FR-patched immunoglobulin chains operably linked to an expression control sequence (including promoter and enhancers) are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Selectable markers such as tetracycline, neomycin, beta-lactamase, etc., are included in the vector to allow detection of cells transformed with the DNA vectors (see, for example, US Pat. No. 4,704,362, which is incorporated herein by reference).

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Bacterial hosts are suitable for propagating the DNA vectors as well as expressing the incorporated immunoglobulin DNA. For example, E. coli is the most commonly used prokaryotic host used for cloning the DNA sequence for the present invention. Other microbial hosts that are useful for the same

- purposes include, as examples, bacilli (for example *Bacillus subtilis*), and other enterobacteriaceae (for example *Salmonella*, *Serratia*), and various *Pseudomonas* species. Expression of cloned sequences in these hosts require the presence of expression control sequences compatible with the host cell
- 5 (for example an origin of replication), and functional promoters to be included in the DNA vector. Example of well-known promoter system include, but not limited to, tryptophan (*trp*) promoter system, beta-lactamase promoter system, phage lambda promoter system, etc. These promoters are responsible for controlling expression, or transcription, of the functional gene sequence
- 10 downstream of the promoter system, which contains, in addition to all necessary motifs, and optionally with an operator sequence, ribosome binding site sequences and the like, necessary for transcription initiation and translation.
- 15 Similarly, other microbes, such as yeast, may also be used for expression. For example, a preferred host will be *Saccharomyces*, which is a suitable host for expression vectors containing the appropriate expression control elements, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as
- 20 desired.
- Eukaryotic host cells of invertebrate origin can be used. For example, insect cells, such as hi-5, SF9, SF21. Appropriate expression vectors containing convenient cloning sites, promoters, termination sequences, etc., that are
- 25 important for high-level expression in the host cells are available commercially (Invitrogen, San Diego, CA).
- Preferably, mammalian tissue cell culture may be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to
- 30 Clones," VCH Publisher, NY, NY, 1987, which is incorporated herein by reference). The most commonly used mammalian host cells are Chinese Hamster Ovary (CHO) cell lines, various COS cell lines, HeLa cells, and myeloma cell lines such as SP2/0 cell lines, NS0 cell lines, YB2/0 cell lines, etc, and transformed B-cells or hybridomas. These cell lines are capable of

- “Protein Purification”, Springer-Verlag, NY, 1982). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may
- 5 then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like (See, generally, Immunological Methods Vols I and II, Lefkovits and Pernis, eds., Academic Press, New York, NY, 1979 and 1981).
- 10 The antibodies of the present invention will typically find use individually, or in combination with other treatment modalities, in treating diseases susceptible to antibody-based therapy. For example, the immunoglobulins can be used for passive immunization, or the removal of unwanted cells or antigens, such as
- 15 by complement mediated lysis, all without substantial adverse immune reactions (for example anaphylactic shock) associated with many prior antibodies.
- A preferable usage of the antibodies of the present invention will be the treatment of diseases using their naked forms (naked antibodies) at dosages
- 20 ranging from 50 mg to 400 mg/m², administered either locally at the lesion site, subcutaneously, intravenously, and intramuscularly, etc. Multiple dosing at different intervals will be performed to achieve optimal therapeutic or diagnostic responses, for example, at weekly intervals, once a week, for four weeks. Usage of the antibodies derived from the present invention can be
- 25 combined with different treatment modalities, such as chemotherapeutic drugs (for example CHOP, Dox, 5-Fu, ..etc), radiotherapy, radioimmunotherapy, vaccines, enzymes, toxins/immunotoxins, or other antibodies derived from the present invention or others. The antibodies of the present invention, if specific for the idiotype of an anti-tumor antibody, can be used as tumor vaccines for the elicitation of Ab3 against the tumor antigen. Numerous additional agents,
- 30 or combinations of agents, well-known to those skilled in the art may also be utilized.

Additionally, the antibodies of the present invention can be utilized in different pharmaceutical compositions. The antibodies can be used in their naked forms, or as conjugated proteins with drugs, radionuclides, toxins, cytokines, soluble factors, hormones, enzymes (for example carboxylesterase, ribonuclease), peptides, antigens (as tumor vaccine), DNA, RNA, or any other effector molecules having a specific therapeutic function with the antibody moiety serving as the targeting agents or delivery vehicles. Moreover, the antibodies or antibody derivatives, such as antibody fragments, single-chain Fv, diabodies, etc. of the present invention can be used as fusion proteins to other functional moieties, such as, antibodies or antibody derivatives of a different invention (for example as bispecific antibodies), toxins, cytokines, soluble factors, hormones, enzymes, peptides, etc. Different combinations of pharmaceutical composition, well-known to those skilled in the art may also be utilized.

FR-patched antibodies of the present invention can also be used for in vitro purposes, for example, as diagnostic tools for the detection of specific antigens, or the like.

The following examples are offered by way of illustration, not by limitation.

EXPERIMENTAL

In designing the amino acid sequence of the FR-patched immunoglobulin chain, the murine variable region sequence (applies to both VH and VL) was compartmentalized into FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4, according to Kabat's classification (Kabat et al., op. cit.). Selection of the individual FR's for patching was in accordance to the guidelines as described previously.

A human FR1 will be used when it has the highest homology to the parent FR1, preferably 100%, at three or more amino acids immediately adjacent to CDR1.

A human FR2 will be used when it has the highest homology to the parent FR2, preferably 100%, at three or more amino acids at both ends immediately adjacent to the flanking CDR1 and CDR2.

- 5 A human FR3 will be used when it has the highest homology to the parent FR3, preferably 100%, at three or more amino acids at both ends immediately adjacent to the flanking CDR2 and CDR3.

- A human FR4 will be used when it has the highest homology to the parent
10 FR4, preferably 100%, at three or more amino acids immediately adjacent to CDR3.

- In case human FR's with 100% homology at three or more amino acids adjacent to the CDRs cannot be identified, FR's with the closest homology at
15 these positions containing conservatively similar amino acids, such as, gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr, will be selected.

- Preferably, human FR's whose amino acids at positions known to be close to,
20 or have interactions with the CDR's/antigen binding site (Chothia and Lesk, op. cit.; Chothia et al., op. cit., and Tramontano et al., op. cit.), either based on computer modeling (see Levy et al., Biochemistry op. cit.; Bruccoleri et al., op. cit.; Chothia et al., op. cit.), crystal structure, published information, or prior experience, which are identical, or conservatively similar to that of the
25 parent FR's will be selected.

- In case where a particular human FR satisfying all the above is unavailable, and direct patching results in the loss of affinity or specificity, murine residues considered to have interactions with the antigen binding site, or contribute to
30 the final affinity of the antibody, will be introduced back to the best available FR. Alternatively, the particular FR with no matching human counterpart will be retained and used in its murine composition without modification; the final FR-patched sequence will contain a mixture of human and murine FR's. For

the purpose of illustration, two murine anti-B cell antibodies will be FR-patched using the approach as described herein in this invention.

EXAMPLE 1

FR-PATCHED ANTI-CD22 ANTIBODY

Design of genes for FR-patched anti-CD22 light and heavy chain

The heavy and light chain sequence of a murine anti-CD22 antibody, RFB4 (Li et al., Cell Immunol. 118:85, 1989; Mansfield et al., Blood 90:2020-2026, 1997) is used as an example to illustrate the approach of using FR-patching to reduce or eliminate immunogenicity of the re-engineered antibody. The sequences of the heavy (a) and light chain (b) variable region for the murine antibody are shown in Figure 1.

Patching of the individual FRs for the heavy chain variable region for RFB4 was done as follows:

- a. FR1: the FR1 sequence of the murine VH was compared with the FR1 sequences of human VH from the Kabat's database (Kabat et al., op. cit.). Although human FR1 of the highest sequence homology is preferred, particular emphasis on the sequence closest to the CDR1 was taken. There are three FR1 sequences that are of high homology to the murine FR1. They are, namely, EIK, RF-SJ1, and WAS. The FR1 with the highest overall homology with the five residues closest to the CDR1 identical to the murine parent is EIK, however, there is a missing residue in position 12, which can create potential problems affecting the immunoreactivity of the resultant antibody. The preferred FR1 picked for the patching was therefore from WAS. First, except at position 28, the third closest residue to CDR1, a whole stretch of 11 amino acids next to the CDR1 is identical to the murine parent. In position 28, a serine residue is found instead of an alanine in the murine sequence. Since serine is considered as a hydroxylated version of alanine, the change is conservative. Moreover, residues that are different between the human and murine are relatively similar in characteristics. For example, valine and leucine in position 5, lysine and glutamine at position 13, lysine and arginine at position 19, and

alanine and serine at position 28. Therefore, human sequence from WAS was chosen for patching the FR1 of the anti-CD22 antibody (Figure 2A).

- 5 b. FR2: by the same token and based on the degree of homology, the human WAS sequence is chosen for patching the FR2 of the anti-CD22 antibody (Figure 2A).
- 10 c. FR3: with the sequences closest to the CDR2, and CDR3 being identical, and the high degree of homology, the human GAL sequence was selected for patching the FR3 of the anti-CD22 antibody (Figure 2A).
- 15 d. FR4: there are many human FR4 with the sequence closest to the CDR3 being identical, and a high degree of homology to the murine parent. In this example, the human DOB sequence was selected for patching the FR4 of the anti-CD22 antibody (Figure 2A).

20 The final design of the FR-patched VH sequence (Figure 3A) for the anti-CD22 antibody is composed of the human WAS FR1 and FR2, and the GAL FR3 and DOB FR4, replacing the original VH FR's of the anti-CD22 antibody. There is no single mutation or re-introduction of murine FR residues in the final design of the FR-patched sequence.

25 Using a similar strategy, the sequence design for the FR-patched light chain (VL) was done as follows:

- 25 a. FR1: human JOH was chosen for patching the FR1 of the murine VL. It has a high degree of sequence homology and the stretch of 8 amino acids adjacent to the CDR1 being identical to the parent sequence (Figure 2B).
- 30 b. FR2: human Vd'CL was chosen for patching the FR2 of the murine VL, for similar reasons. More than 4 identical sequences are adjacent to the CDR1, and CDR2 (Figure 2B).
- 35 c. FR3: human WES was chosen for patching the FR3 of the murine VL. FR3 has the longest sequence, and the sequence homology between WES

and the murine FR3 is high, with the sequences flanking the CDR2 and CDR3 being identical (Figure 2B).

- d. FR4: human RZ was chosen for patching the FR4 of the murine VL, for similar reasons (Figure 2B).

The final design of the FR-patched VL sequence (Figure 3B) for the anti-CD22 antibody is composed of the human JOH FR1, Vd'CL FR2, WES FR3, and RZ FR4, replacing the original VL FR's of the anti-CD22 antibody. Once again, there is no single mutation or re-introduction of murine FR residues in the final design of the FR-patched sequence.

Construction of the FR-patched heavy and light chain genes

The designed heavy and light chain variable region sequences of the FR-patched antibody are assembled by a combination of oligonucleotide synthesis and PCR using a variety of published methods (Leung et al., op. cit.; Daugherty et al., op. cit.; DeMartino et al., op. cit.; Jones et al., op. cit.).

To construct the FR-patched heavy chain variable region sequence (SEQ ID no. 1), the full DNA sequence is divided into two halves: the N-terminal half and the C-terminal halves. Both were constructed separately by PCR and the complete variable region sequence was formed by joining the N- and C-terminal halves at the KpnI site.

The N-terminal half was constructed as follows: a N-template (SEQ 3) is a synthetic sense-strand oligonucleotide (111-mer) encoding amino acid 14 – 50 of the VH region (SEQ ID no. 2). The template is PCR-amplified by two primers:

The 5' Primer (SEQ ID no. 4) is a synthetic sense-strand oligonucleotide (57-mer) encoding amino acid 1 – 19 of the VH region. The 3' end of the primer overlaps with the 5' end of the template by 18 nucleotides.

The 3' Primer (SEQ ID no. 5) is a synthetic anti-sense-strand oligonucleotide (48-mer) encoding amino acid 43 – 59. The primer overlaps with the template by 21 nucleotides.

- 5 The N-template (SEQ ID no. 3) was PCR-amplified using the 5' and 3' primer set (SEQ ID no. 4 & 5) using standard techniques and procedures.

- The C-terminal half was constructed as follows: a C-template (SEQ ID no. 6) is a synthetic sense-strand oligonucleotide (132-mer) encoding amino acid 68 – 111 of the VH region (SEQ ID no. 2). The template was PCR-amplified by two primers:
- 10

- The 5' Primer (SEQ ID no. 7) is a synthetic sense-strand oligonucleotide (60-mer) encoding amino acid 55 – 74 of the VH region. The 3' end of the primer overlaps with the 5' end of the template by 21 nucleotides.
- 15

- The 3' Primer (SEQ ID no. 8) is a synthetic anti-sense-strand oligonucleotide (58-mer) encoding amino acid 105 – 123 of the VH region. The primer and the template overlaps by 21 nucleotides.
- 20

- The C-template (SEQ ID no. 6) is PCR-amplified using the 5' and 3' primer set (SEQ ID no. 7 & 8) using standard techniques and procedures.

- For the construction of the full-length FR-patched RFB4 VH domain, the N-template (SEQ ID no. 3, 111-mer), C-template (SEQ ID no. 6, 132-mer), and their respective 5'- and 3' primers (SEQ ID no. 4 & 5 for N-template, and SEQ ID no.7 & 8 for C-template), were synthesized on an automated Applied Biosystem 380B DNA synthesizer (Foster City, CA). The oligonucleotides were desalted by passing through a CHROMOSPIN-10TM column (Clonetechn, Palo Alto, CA). The oligonucleotides were adjusted to a final concentration of 20 µM. One µl of template oligonucleotides at various dilutions (10X, 100X, 1000X and 10000X, etc.) were mixed with 5 µl of their corresponding flanking primers in the presence of 10 µl of 10x PCR Buffer (500 mM KCl,
- 25
- 30

oligonucleotides were adjusted to a final concentration of 20 μ M. One μ l of template oligonucleotides at various dilutions (10X, 100X, 1000X and 10000X, etc.) were mixed with 5 μ l of their corresponding flanking primers in the presence of 10 μ l of 10x PCR Buffer (500 mM KCl, 100 mM Tris.HCl buffer, pH 8.3, 15 mM MgCl₂) and 5 units of AMPLITAQ™ DNA polymerase (Perkin Elmer). This reaction mixture was adjusted to a final volume of 100 μ l and subjected to 30 cycles of PCR reaction consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minutes, and polymerization at 72°C for 1 minute. The PCR reaction mixtures were analyzed under 2% agarose gel electrophoresis. The highest template dilution that gave rise to sufficiently abundant product of the right size would be chosen for further processing.

Double-stranded PCR-amplified products for the N- and C-templates were gel-purified, restriction-digested with SpeI. The restricted N- and C-double stranded DNA were ligated at the SpeI site, and the ligated products were subjected to another round of PCR-amplification using the 5' primer for the N-template (SEQ ID no.12) and the 3' primer for the C-template (SEQ ID no. 16). The PCR product with a size of ~320 was directly cloned into the TA cloning vector (Invitrogen). The sequence of the cloned fragment was confirmed by Sanger's method (Sanger op. cit.) to be identical to the designed VL sequence. The confirmed sequence was used to replace the VL sequence of a light chain expression vector containing an IgH promoter, an Ig enhancer, a human kappa constant region genomic sequence, and a selectable marker, hyg. The final light chain expression vector is designated as hpRFB4pSMk.

Expression and affinity of FR-patched antibody

The expression plasmids hpRFB4pSMh and hpRFB4pSMk were linearized and co-transfected into mouse Sp2/0 cells. Cells transfected with the plasmids were selected in the presence of mycophenolic acid and/or hygromycin B conferred by the gpt and hyg genes on the plasmids by standard methods. Cells surviving selection were tested for human antibody secretion using ELISA methods. Clones that were identified to be secreting human antibody

- c. FR3: human heavy chain FR3 sequences with satisfactorily high sequence homology and identical sequences adjacent to the CDR2 and CDR3 could not be identified. Although the human FR3 from 783C¹CL exhibited 78% of sequence homology, the residues flanking the CDR2 were drastically different, despite the differences being conserved. For example, the K, A, and L at positions 57, 58 and 60 (Kabat's numbering, Kabat et al., op. cit.) which are the 1st, 2nd, and 4th residues closest to the CDR2 are replaced by the conserved human residues R, V and I, respectively. Nevertheless, the high number of changes in proximity to the CDR2, albeit conservative, could result in significant conformational changes at the antigen binding site. Without risking the loss of affinity, and as an illustration on the flexibility of the FR-patching approach, the FR3 would not be patched with any of the human FR's. Instead, the murine FR3 sequence was retained in this particular antibody (Figure 8A).
- d. FR4: there are many human FR4 with the sequence closest to the CDR3 being identical, and a high degree of homology to the murine parent. In this example, the human 4G12¹CL sequence was selected for patching the FR4 of the anti-CD20 antibody (Figure 8A).

The final design of the FR-patched VH sequence (Figure 9A) for the anti-CD20 antibody is composed of the human LS2'CL FR1, NEWM FR2, murine 1F5 FR3 and 4G12'CL FR4, replacing the original VH FR's of the murine anti-CD20 antibody.

An alternative design would be a patched VH containing the murine CDRs embedded in human LS2'CL FR1, NEWM FR2, 783C'CL FR3, and 4G12'CL FR4 (Figure 10A). For the purpose of illustration, the construction of the former version would be described below.

Using a similar strategy, the sequence design for the FR-patched light chain was constructed as follows:

- a. FR1: human BJ19 was chosen for patching the FR1 of the murine VL. This is the human FR1 sequence with the highest homology to the murine parent (61%). Moreover, some of the human residues that are different from that of the murine are conserved. For example, the E to D, and K to R conversions at positions 18 and 19, respectively, are conserved changes (Figure 8B).
5
- b. FR2: although there is a human FR2, MOT, that was found to be of high sequence homology (73%) to the murine FR2, the Tryptophan at position 32 (Kabat's numbering, Kabat et al., op. cit.), the 3rd residues closest to the CDR2, was replaced by a non-conservative Valine in the MOT FR2 sequence. This replacement potentially might have a significant effect on the final conformation of the antigen binding site. It was therefore determined that the murine FR2 of the VL domain would remain in the design of the FR-patched antibody (Figure 8B).
10
15
- c. FR3: human WES was chosen for patching the FR3 of the murine VL. FR3 has the longest sequence, and the sequence homology between WES and the murine FR3 is 71%, with the three human residues flanking the CDR2 and CDR3 being identical to that of the murine (Figure 8B).
20
- d. FR4: human λ FR4 sequence from NIG-58 was chosen for patching the FR4 of the murine VL, for similar reasons. The sequences are 72% homologous to the stretch of 7 residues adjacent to the CDR3 being identical between the human and murine (Figure 8B).
25

The final design of the FR-patched VL sequence (Figure 9B) for the anti-CD20 antibody is composed of the human BJ19 FR1, murine 1F5 FR2, WES FR3, and NIG-58 FR4, replacing the original VL FR's of the anti-CD20 antibody. An alternative design of FR-patched VL would be composed of the human BJ19 FR1, MOT FR2, WES FR3, and NIG-58 FR4, forming the scaffold supporting the CDR loops (Figure 10B). For the purpose of
30

illustration in this application, only the construction of the former FR-patched VI. would be described below.

Construction of the FR-patched heavy and light chain genes

The designed heavy and light chain variable region sequences of the FR-patched antibody were assembled by a combination of oligonucleotide synthesis and PCR using a variety of published methods.

To construct the FR-patched heavy chain variable region sequence (SEQ ID no. 17), the full DNA sequence was divided into two halves. The N-terminal half and the C-terminal half were constructed separately by PCR and the complete variable region sequence is formed by joining the N- and C-terminal halves at the SpeI site.

The N-terminal half was constructed as follows: a N-template (SEQ ID no. 19) is a synthetic sense-strand oligonucleotide (114-mer) encoding amino acid 12 - 49 of the VH region (SEQ ID no. 18). The template is PCR-amplified by two primers:

The 5' Primer (SEQ ID no. 20) is a synthetic sense-strand oligonucleotide (57-mer) encoding amino acid 1 - 19 of the VH region. The 3' end of the primer overlaps with the 5' end of the template by 24 nucleotides.

The 3' Primer (SEQ ID no. 21) is a synthetic anti-sense-strand oligonucleotide (55-mer) encoding amino acid 43 - 60. The primer overlaps with the template by 21 nucleotides.

The N-template (SEQ ID no. 19) is PCR-amplified using the 5' and 3' primer set (SEQ ID no. 20 & 21) using standard techniques and procedures.

The C-terminal half was constructed as follows: a C-template (SEQ ID no. 22) is a synthetic sense-strand oligonucleotide (126-mer) encoding amino acid 70

– 111 of the VH region (SEQ ID no. 18). The template was PCR-amplified by two primers:

5 The 5' Primer (SEQ ID no. 23) is a synthetic sense-strand oligonucleotide (61-mer) encoding amino acid 57 – 76 of the VH region. The 3' end of the primer overlaps with the 5' end of the template by 21 nucleotides.

10 The 3' Primer (SEQ ID no. 24) is a synthetic antisense-strand oligonucleotide (59-mer) encoding amino acid 105 – 123 of the VH region. The primer and the template overlaps by 21 nucleotides.

The C-template (SEQ ID no. 22) was PCR-amplified using the 5' and 3' primer set (SEQ ID no. 23 & 24) using standard techniques and procedures.

15 For the construction of the FR-patched 1F5 VH domain, the N-template (SEQ ID no. 19, 114-mer), C-template (SEQ ID no. 22, 126-mer), and their respective 5'- and 3' primers (SEQ ID no.20 & 21 for N-template, and SEQ ID no.23 & 24 for C-template), were synthesized on an automated Applied Biosystem 380B DNA synthesizer. The oligonucleotides were desalted by
20 passing through a CHROMOSPIN-10TM column (Clonetech). The oligonucleotides were adjusted to a final concentration of 20 µM. One µl of template oligonucleotides at various dilutions (10X, 100X, 1000X and 10000X, etc.) were mixed with 5 µl of their corresponding flanking primers in the presence of 10 µl of 10x PCR Buffer (500 mM KCl, 100 mM Tris.HCl
25 buffer, pH 8.3, 15 mM MgCl₂) and 5 units of AMPLITAQTM DNA polymerase (Perkin Elmer). This reaction mixture was adjusted to a final volume of 100 µl and subjected to 30 cycles of PCR reaction consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 1.5 minutes, and polymerization at 72°C for 1 minute. The PCR reaction mixtures were
30 analyzed under 2% agarose gel electrophoresis. The highest template dilution that gave rise to sufficiently abundant product of the right size would be chosen for further processing.

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5 Double-stranded PCR-amplified products for the N- and C-templates were gel-purified, restriction-digested with SpeI site. The N- and C-double stranded DNA were ligated at the BspEI site, and amplified using the 5' primer for the N-template (SEQ ID no.12) and the 3' primer for the C-template (SEQ ID no. 16). The PCR product with a size of ~320 was directly cloned into the TA cloning vector (Invitrogen). The sequence of the cloned fragment was 10 confirmed by Sanger's method (Sanger et al., op. cit.) to be identical to the designed VL sequence. The confirmed sequence was used to replace the VL sequence of a light chain expression vector containing an IgH promoter, an Ig enhancer, a human kappa constant region genomic sequence, and a selectable marker, hyg. The final light chain expression vector is designated as 15 hp1F5pSMk.

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